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AUTHOR(S):

Mitsuda, Hisateru; Shikanai, Takehiko

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Studies on the Utilization of Chlorella for Food*

Studies on the Nutritive Values of Cell Free Algal Proteins

Hisateru MITSUDA and Takehiko SHIKANAI**

(Laboratory of Nutritional Chemistry, Faculty of Agriculture, Kyoto University)

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It is desirable to utilize the chlorella not only for a resource of food protein but for the resources of pigments such as chlorophyll, carotine, and other resources.

For this purpose, the butanol treatment or autolysis-butanol treatment was the most available method ; by these methods protein, pigments, and other soluble materials were isolated simultaneously. By the autolysis-butanol treatment, the cell free algal protein was prepared, and then, its digestibility and biological value were investigated.

The digestibility of the cell free protein was much higher than that of dried or decolored algae. It can match with casein in digestibility.

The biological value of the cell free protein, however, was somewhat lower than that of casein. But the isolated protein could be used for the improvement of nutritive value of cereal proteins. This was explained by the circular explanatogram of limiting amino acid which was devised by the authors.

INTRODUCTION

At first, the utilization of unicellular green algae such as chlorella, scenedesmus for food was picked up by German chemists during the First War, aiming to solve the shortage of foods. But this was not developed into regular researches.

During the Second War, in America, chlorella was noticed as a source of antibacterial substance that might be isolated from the culture solution of chlorella[1]. It has given a solution that the antibacterial activity was a result of a complex photo-oxidation of the unsaturated fatty acids in the chlorella itself, and similar reaction could be induced in fatty acids from a variety of source[2)3)].

After the War, the investigation of the influence of environment on the chemical composition of chlorella was resumed. H. A. Spoehr and H. W. Millner studied the change of chemical compositions accompanied by the change in the circumstancial conditions[4)5)], and confirmed the possibility of growing chlorella on a large scale as food[6)].

Since that time, the studies on the mass culture of these unicellular green algae were started in various countries, including U. S. A.

In Japan, especially in the Tokugawa Instiute for Biological Research, the

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** 満田久輝, 鹿内健彦

researches for the mass culture of chlorella and scenedesmus were pursued by H. Tamiya and his coworkers, aiming the development of the food protein source[7)8)].

They obtained the results that, by means of the out-door open circulating method, they were producing the yields of chlorella or scenedesmus of 15-19 g. per day per square meter (averaged) on a pilot plant scale[9)].

The researches on nutritive values of these algae as food materials are continued in various countries. One of the most considerable results of them was given by H. Fink[10)11)]. According to his rat feeding tests, the dried scenedesmus, which gave the digestibility of 60 per cent, has a nutritive value equal to that of milk protein.

On the other hand, in Japan, the Research Council for the Studies on Essential Amino Acid pursued the researches of the nutritive values of the protein of dried algae. But satisfactory results were not obtained. Owing to the hardness of the algal cell wall, the digestibilities of dried algal proteins were very low, and they caused diarrhea or some other troubles. Moreover, dried algae themselves did not suit to human tastes by their dark greenish color, which came from their extremely high contents of chlorophyll, and their unpleasant flavors. On this account, many attempts have been made to measure the nutritive value of decolored algae by means of hot methanol extraction. However satisfactory results on their digestibilities have not been odtained yet.

For the utilization of these algae as a food protein source, the followings must be pursued ; these are, to remove the cell wall which cause the indigestion, to reform the algal protein into easily intakable food material on the point of color and flavor, and still more, to isolate effectively the precious vitamins, such as folic acid and vitamin B_{12}, and pigments, such as chlorophyll, carotine and other active substances. Then, the perfect utilization of algal components should be attempted.

On this purpose the studies on effective fractional isolation of chlorella components were carried out, and it was recognized that the treatment of algae with butanol was one of the most effective methods. By this treatment, a considerable amount of the cell free algal protein was isolated from 5 kg. of fresh scenedesmus, and its digestibility and biological value were investigated.

In this paper, the results of these experiments are reported and the nutritive value of algal protein and possibility for a food are explained by using the circular explanatogram of limiting amino acid which was devised by the authors.

I. THE DESTRUCTION OF ALGAL CELL WALL

Materials and methods

Algae. These were the fresh and lyophilized chlorella (scenedesmus) supplied from the Tokugawa Institute for Biological Research.

Nitrogen assay. Total nitrogen extracted was determined as follows. The algal paste was suspended into 10 volume of the solvent, stirred at a specified temperature (when they were treated by the procedure such as grinding freezing, and others, after these procedures were done). Then it was centrifuged and the nitrogen content of one part of the supernatant solution was measured.

The protein-nitrogen was assayed by the following method. A part of the supernatant described above was adjusted to pH 3.5 with hydrogen chloride or acetic acid, heated to 70° for 30 minutes, and the sedimented protein was collected by centrifugation. Its the nitrogen content was measured, and thus the protein-nitrogen was determined.

As for the lyophilized algae, they were previously kneaded with 3 volume of water, and then brought to the similar condition as the fresh algae, and then to the same treatment as the fresh algae.

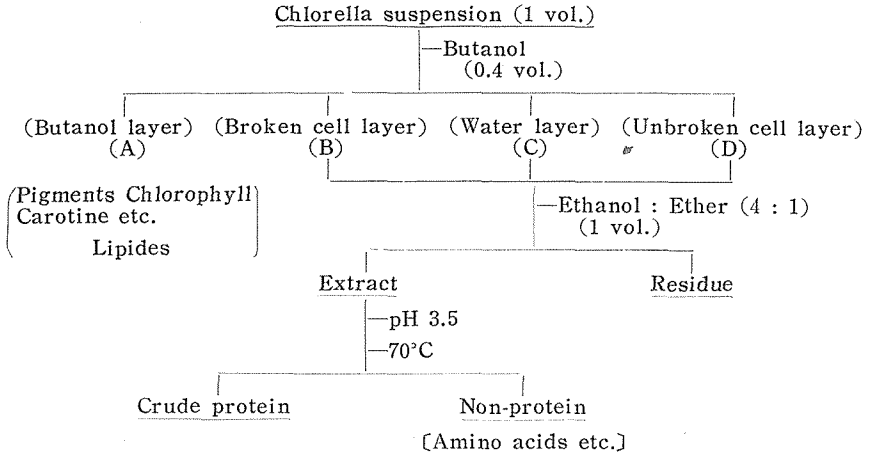
All of the nitrogen assays were carried out by the semi-micromethod of A.O.A.C.

Treatments of algae. The effects of various treatments of destracting the cell wall described in Table 1 were investigated. The procedures of treatments of them were as follows.

a) Butanol treatment. The procedure was summarized in Scheme 1, that is, a 0.4 volume of *n*-butanol (iso-butanol) was added dropwise into the algal suspension, they were stirred, and then centrifuged. The suspension gave 4 layers : from above, butanol layer (A), broken cell layer (B), water layer (C), and unbroken cell layer (D). The butanol layer contained lipids or pigments such as chlorophyll, carotine, and other like. In water layer 16 per cent of the total algal nitrogen was extracted, but the larger part of nitrogenous materials remained in the cell and went into layers B and D.

After removing the whole part of the butanol layer (A), the other 3 layers (B, C, and D) were mixed and an ethanol-ether mixture (4 : 1) of a volume equal to the original volume of water in the algal suspension was added

Scheme 1. The summary of butanol treatment.



dropwise into this mixture and stirred. Then the cell particles such as chloroplast and the others were dispersed into medium solution, and thus more nitrogenous materials were extracted.

b) Autolysis. Fresh algal paste or suspension was mixed with equal volume of toluol, mixed thoroughly, incubated at 37-38° for a definite time, mixed with 10 volume of water or other solvent and then stirred at room temperature. The extract was separated from the residue by means of centrifugation.

c) Autolysis-butanol treatment. At first, autolysis was done by the method described above, they were suspended into 10 volume of water (Scheme 1) and thus the butanol treatment was done.

Results

The results of extractions by the various methods are summarized in Table 1. The extractability was indicated by the percentage to total algal

Table 1. The extractabilities of algal protein by various treatments.

Expt. No.	Treatment	Fresh Chlorella			Lyophilized Chlorella		
		Extract		Residual-N (%)	Extract		Residual-N (%)
		Protein-N (%)	Non-protein-N (%)		Protein-N (%)	Non-protein-N (%)	
1	Water extraction	0	0	100.0	3.2	11.0	83.0
2	Hot water extraction				6.5	16.1	77.4
3	Balle mill treatment, water extraction				5.6	15.9	68.3
4	Grinding, water extraction	4.7	7.5	87.8	3.4	16.1	70.5
5	Grinding, 0.1% NaOH extraction	6.6	14.7	78.7			
6	Grinding, 1.0% NaOH extraction	20.5	18.1	61.4			
7	Phosphate buffer extraction, pH 7.0		1.4	97.3	7.2	9.3	83.5
8	Phosphate buffer extraction, pH 10.0	1.4	2.1	96.5			
9	Freezing-Melting with liquid air, water extraction	21.0	43.0	36.0			
10	Freezing-Melting with liquid air, hot water extraction	7.0	24.2	68.6			
11	Alkaline extraction, 0.1% NaOH				20.5	10.7	68.5
12	Alkaline extraction, 0.5% NaOH	22.1	8.1	69.8	23.5	14.0	62.5
13	Alkaline extraction, 0.1% NaOH (24 hrs.)				25.3	13.4	61.6
14	Alkaline extraction, 0.5% NaOH (24 hrs.)				29.5	19.5	51.6
15	Butanol treatment	25.1	17.9	57.0			
16	Autolysis (toluol 1:1, 37°, 24 hrs.)	17.5	25.0	57.5	7.5	22.2	70.3
17	Autolysis-Butanol treatment	23.6	36.4	40.0			

Temp. and time of extraction without description was room temp. and 30 min..

nitrogen. From this table, the followings were concluded.

1) If they were free from some pretreatment, nitrogenous cell materials could not be extracted from the fresh algae only by water or buffering solu-

tion. This was natural from the point of view of biological state of the algae.

2) Grinding as a means of pretreatment (for 3 hours, with one third weight of sea sand) was useful in increasing the extractability. The repetition of freezing-melting by liquid air could extract 64 per cent of total algal nitrogen by water extraction at room temperature. From the above results, breakage of the cell wall by means of such a mechanical treatment as grinding or freezing-melting was found to be an effective means for the extraction of nitrogenous cell materials.

3) Extraction by using alkali solution was very effective ; even the dilute alkali-solution as low as 1 per cent could extract 30-50 per cent of nitrogen at room temperature. When it was treated at higher temperature or more concentrated alkali solution was used, nearly the whole amount of algal nitrogen could be extracted. But the proteins obtained by the alkaline extraction had a thick color, and coloring matter could not be extracted by the organic

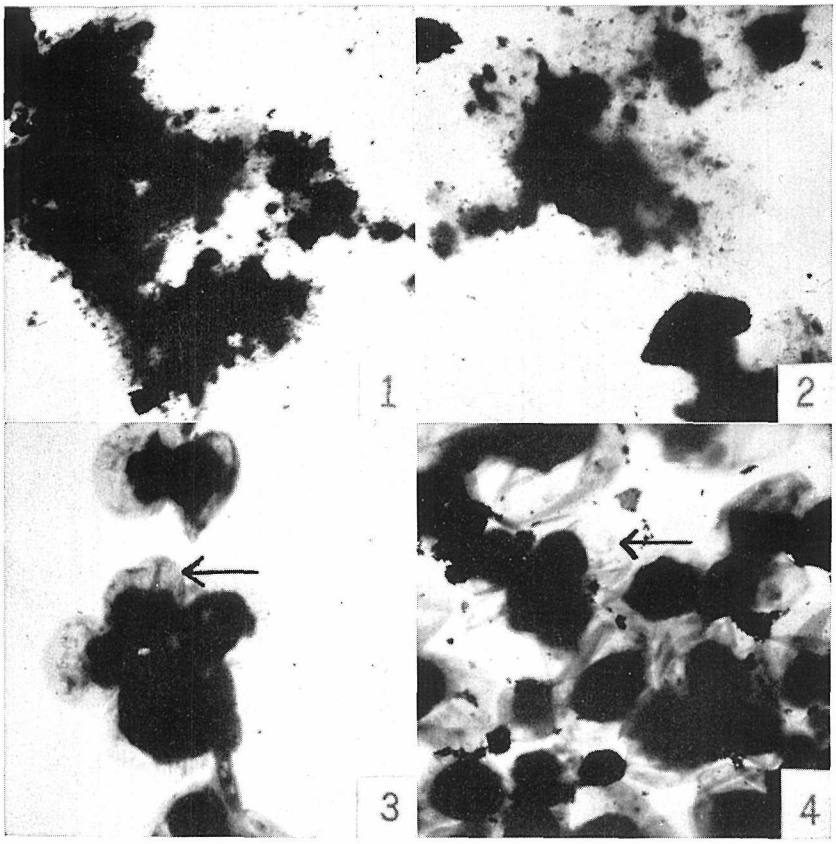


Fig. 1. The electromicrographs of chlorella proteins.
1) The isolated cell free chlorella protein with the butanol treatment.
2) The isolated cell free chlorella protein treated mildly with trypsin.
3) The extracted residue of chlorella with the butanol treatment.
4) The decolored chlorella treated with trypsin.
The arrows indicate the presence of cell wall (parts of pale shadows).

solvents such as alcohol, acetone, and the like. Hence, they had only poor utility as a food protein.

4) Extraction by the direct butanol treatment (without autolysis) was effective and their results were match for that of freezing-melting treatment or that of diluted alkaline extraction. Protein-nitrogen extracted by this treatment was 25 per cent of the total algal nitrogen and 29 per cent of total true protein-nitrogen (86 per cent of total nitrogen of used algae was protein-nitrogen). By this treatment, as the authors planned, the effective fractional isolation not only of protein but of non-protein-nitrogenous materials, and pigments such as chlorophyll, carotine and so on, was possible. Therefore, this treatment was suitable for the purpose of complete utilization of algae.

Of course, the protein obtained by this method was a completely cell free preparation. This was comfirmed by electromicroscopic observations (Fig. 1).

5) Autolysis was very different from the mechanical or chemical treatment as described above. The results of this treatment strongly depended on the incubation time and temperature (Fig. 2). Moreover, they depended upon

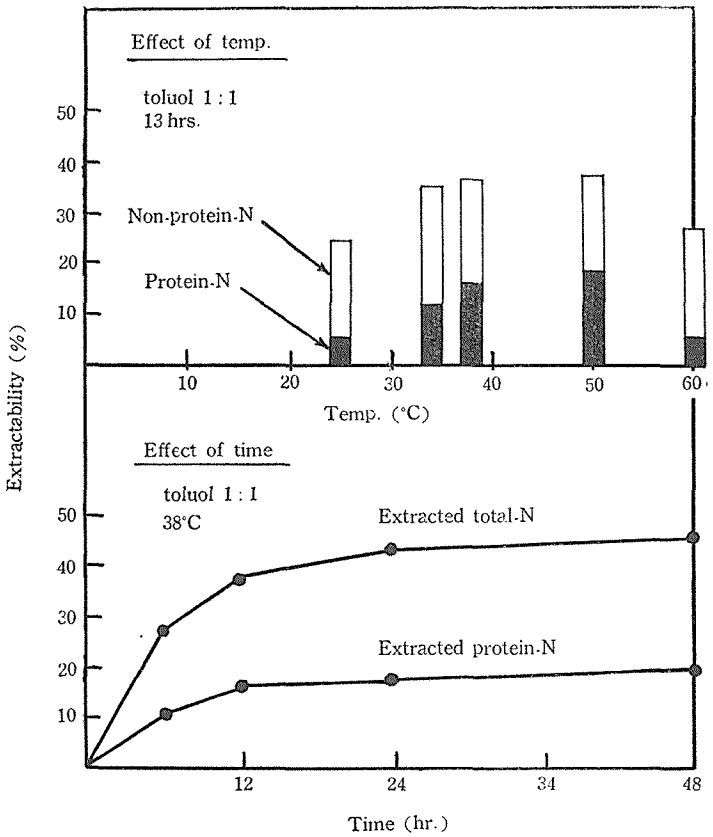


Fig. 2. Effects of temperature and time on autolysis.

the conditions of storage of algae. The longer the period of storage (Table 2), the more nitrogenous materials could be extracted. The extractability of protein-nitrogen of lyophilized algae was not influenced by autolysis, but the

extractability of nonprotein-nitrogen increased a little. (Table 1, Nos. 1, 16 ; Table 2, Nos. 6, 7).

In the autolysis, the longer the incubation time, the more nitrogenous

Table. 2. The extractabilities of algal protein by autolysis.

Expt. No.	Algae	Condition of autolysis	Extractability (%)		Note
			Protein-N	Non-protein-N	
1	Fresh chlorella stocked in ice box for 3 days	Paste,* for 24 hr.	7.5	16.0	
2	for 7 days		10.2	15.0	
3	Fresh chlorella stocked in ice box for 3 days	Paste, for 24 hr.	5.8	12.0	
4	for 7 days	Suspension,** for 24 hr.	3.7	14.0	
5	Fresh chlorella freezed for 1 day and stocked in ice box for 14 days	Paste, for 24 hr.	17.5	25.0	Table 1, No. 16
6	Lyophilized chlorella	Paste, for 24 hr.	7.5	22.2	Table 1, No. 16
7	〃	Suspension, for 24 hr.	6.8	22.2	Table 1, No. 16
8	Fresh scenedesmus, stocked in ice box for 2 days	Paste, for 24 hr.	3.8	14.1	
9	for 10 days		7.6	14.0	
10	stocked in freezed state for 30 days		2.6	17.8	

* Paste ; Algal paste was mixed with equal volume of toluol.
** Suspension ; Algal paste was mixed with 4 volume of phosphate buffer (pH 7.0) and 1 volume of toluol.
Temp. of autolysis ; 37°.

material was extracted. However, as the protein itself was degradated, the yield of protein was decreased.

6) The combination of autolysis and butanol treatment increased the extraction of non-protein-nitrogen, but the yield of protein was not the same.

Discussion

The maximum yields of protein-nitrogen and non-protein-nitrogen were fit for 25 per cent and 60-70 per cent of total algal nitrogen, respectively.

According to the results obtained by L. Fowden[12], the 70 per cent ethanol insoluble nitrogen of algae corresponded to the true protein-nitrogen of algae. Therefore, the true protein-nitrogen of algae used in the experiments were fit for 70-90 per cent of total algal nitrogen. Then, by the methods described above, nearly the whole amount of total nitrogen could be extracted, but the protein was only extracted in yield of about 30 per cent of total true algal protein ; the remaining nitrogen of 70 per cent went into the non-protein-nitrogen fraction.

L. Fowden extracted nearly the whole amount of algal cell contents by means of shearing stress method ; the present authors could obtain similar results by means of alkaline extraction with concentrated sodium hydroxide.

According to these results and to those of electromicroscopic observations, it was expected that the complete extraction of algal nitrogen could hardly be accomplished by the mild treatment as described in Table 1. Moreover, there will be a limit of the extractability, and hence, the values of 60-70 per cent which were given by the present experiments seemed to be the limiting values.

Therefore, in order to extract nearly whole amount of algal nitrogenous materials, the vigorous treatments of destroying the cell wall by the methods used by L. Fowden[12] or D. H. Northcote[13], might be carried out.

II. DIGESTIBILITY AND BIOLOGICAL VALUE OF CELL FREE ALGAL PROTEIN

Methods and Materials

Preparation of cell free algal protein. As described above, the butanol or autolysis-butanol treatment was one of the most effective treatments for the fractional extraction of algal cell materials. But these methods had one difficulty, that is, the use of ethanol-ether mixture was not suitable for the treatment of a large amount of algae. This difficulty was avoided by using the grinding procedure instead of mixing with the solvent. This altered butanol treatment gave the same yield of protein as that obtained by the original method.

4.7 kg of fresh scenedesmus were treated by this altered method, and about 120 g. (dry weight) of cell free protein were prepared (Scheme 2).

Estimation of digestibility of cell free algal protein by trypsin. 100 mg. of dried protein were soaked into 10 ml. of phosphate buffer (0.05 *M*, pH 7.8). and kept in an ice box for 6 hours, in order to swell (or dissolve) the protein. Then, 1 ml. of trypsin solution (4 mg. of crystalline trypsin dissolved in 10 ml. of 0.05 *M*. phosphate buffer pH 7.8) and a few drops of toluol were added. These mixtures were incubated for 24 hours, at 37-38°. After the incubation, 2.5 ml. of 10 per cent trichloroacetic acid were added, kept standing in an ice box for one night, and the sedimented undigested protein was removed by means of filtration. By measuring the nitrogen content of the filtrate, the digested nitrogen was determined.

The control experiments were carried out as follows. The procedure was essentially the same as before, except that the trichloroacetic acid was added before addition of trypsin. Digestibility was given by the next formula.

$$\text{Digestibility } (\%) = \frac{B-b}{A-a} \times 100,$$

where

A : Total nitrogen of sample.

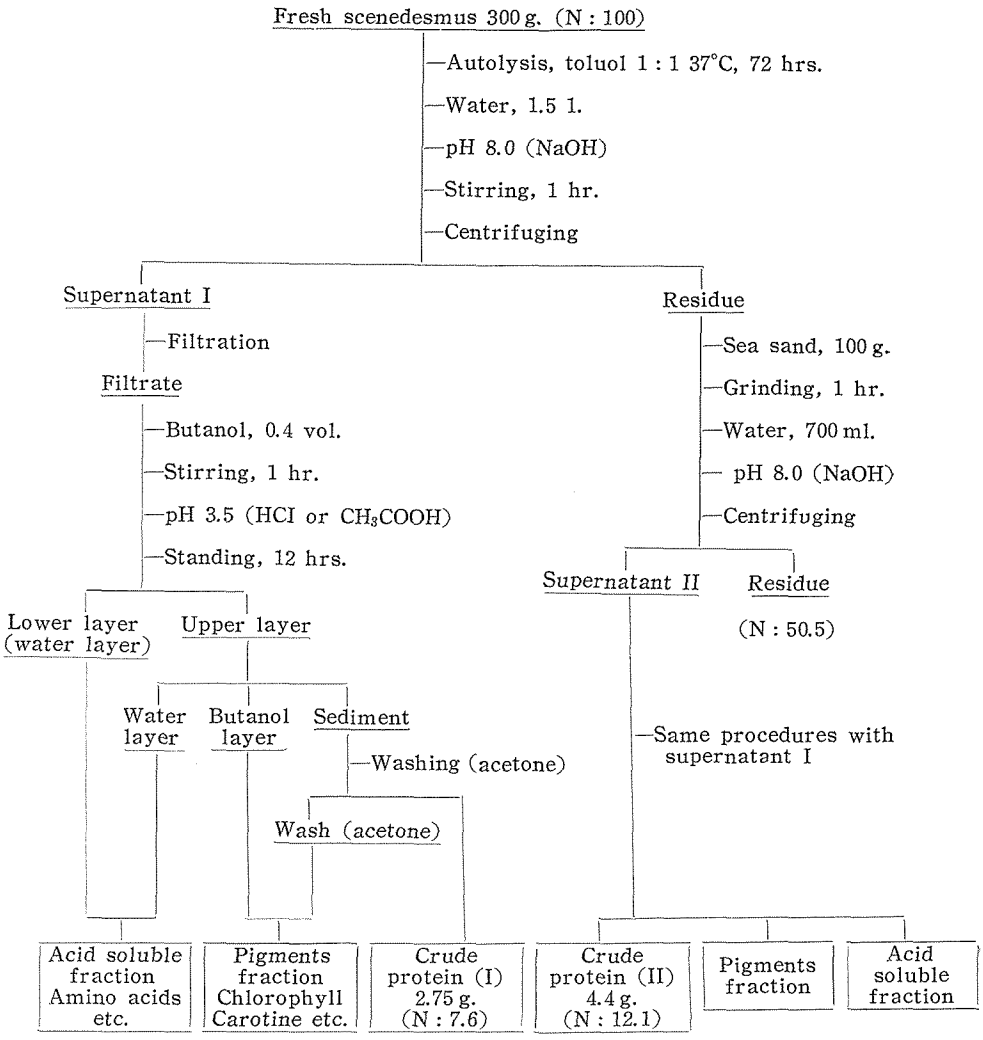
a : Trichloroacetic acid soluble nitrogen of sample.

B : Trichloroacetic acid soluble nitrogen after the trypsin digestion.

b : Trichloroacetic acid soluble nitrogen of control.

The concentration of trypsin was decided by the use of casein as a protein

Scheme 2. The standard procedure of isolation of cell free algal protein.



under the same condition. The use of over 0.4 mg of trypsin gave the same results.

The samples used in this experiment were the above cell free scenedesmus protein and the cell free chlorella protein obtained by means of autolysis-butanol treatment. The dried chlorella, hot methanol treated chlorella, and purified casein were also used for comparison.

Estimation of digestibility and biological value of cell free algal protein by rat feeding tests. The estimation of digestibility and biological value of algal protein by rat were carried out by Mitchell's method[14].

9 female adult rats of wistar strain were grouped into 3 different diet feedings. The lst groups were fed by casein containing diets (control group), the 2nd by cell free algal protein diets, and the 3rd by dried chlorella.

Each groupes were fed for 9 days by non-protein diets, followed by test

Table 3. The diets compositions.

Components \ Protein source	Non protein	Casein	Isolated cell free scenedesmus protein	Dried chlorella
Starch	58 g.	48 g.	48 g.	42 g.
Glucose	30 g.	30 g.	30 g.	30 g.
Protein	10 g.	10 g.	10 g.	18 g.*
Soy-bean oil	5 g.	5 g.	5 g.	5 g.
Vitamin solution	1 ml.	1 ml.	1 ml.	1 ml.
Salt mixture	4 g.	4 g.	4 g.	4 g.
Fiber	2 g.	2 g.	2 g.	0 g.

* 10 g. as the protein.

Salt mixture.

$Ca_3(PO_4)_2$	10 (g.)
K_2HPO_4	37
NaCl	20
$MgSO_4$	8
$Na_2M_0O_4 \cdot 2H_2O$	0.11
Na-Citrate	15
Fe-Citrate	2
Ca-Lactate	8

Vitamin solution (in 1 ml.)

Choline chloride	70 (mg.)	Biotin	0.01 (mg.)
V.B_1-HCl	0.5	Folic acid	0.2
Nicotinamide	2	Inocitol	10
V.B_2	3	V.B_{12}	0.02
V.B_6-HCl	0.6	V.A	2000 I.U.
Ca-pantothenate	0.6	V.D	500 I.U.

diet feedings for 9-10 days.

The diets compositions of each periods were summarized in Table 3. Nitrogen assay of daily urine and feces were carried out for 8 days during the each feeding periods in which the excretion of urine indicated nearly steady states. The daily nitrogen intakes were also determined.

The digestibility and biological value were calculated from the following formulas :

$$\text{Digestibility } (\%) = \frac{\text{Absorbed nitrogen}}{\text{Nitrogen intakes}} \times 100$$

Absorbed nitrogen = (Nitrogen intakes) − (Food nitrogen in feces)

Food nitrogen in feces = (Fecal nitrogen) − (Metabolic nitrogen in feces)

$$\text{Biological value} = \frac{\text{Retention nitrogen}}{\text{Absorbed nitrogen}} \times 100$$

Retention nitrogen = (Absorbed nitrogen) − (Food nitrogen in urine)

Food nitrogen in urine = (Urinary nitrogen) − (Endogenous nitrogen)

Results

The results of the preparation of cell free protein were summarized in Table 4. Considerable differences were observed between the yields of present preparations and the value calculated from the yield of standard procedure described in Scheme 2. That is, in the order of treatment number the yields of crude protein were increased. Especially, in the treatments of the 6 th and the 7 th, the increases in the yields of protein were considerable. The 8 th treatment was carried out at 2 months after the lst treatment. As the storage

Table 4. The yields of cell free scenedesmus proteins.

Treatment No.	Treated algal weight (g.)	Calculated yield* (g.)		Experimental yield (g.)	
		Crude protein I	Crude protein II	Crude protein I	Crude protein II
1	600	5.50	8.80	5.5	8.8
2	600	5.50	8.80	5.0	6.8
3	700	6.42	10.25	8.8	7.0
4	700	11.92	19.05	9.2	18.8
5	600				
6	600	10.16	17.06	28.2	10.3
7	600				
8	300	2.25	4.40	2.3	1.8
Sum up	4700	49.59	78.94	59.0	53.5
		128.53		112.5	

* Calculated from the yield of standard procedure with 300 g. of algae.

Table 5. The digestibilities of algal proteins with trypsin *in vitro*.

Sample	Total-N in sample (A) (mg.)	TCA insoluble-N in Sample (A-a) (mg.)	Added trypsin (mg.)	TCA soluble-N after digestion (B) (mg.)	Digested-N (B-b) (mg.)	Digestibility (%)
Casein 100 mg.	14.30	13.85	0.4	14.50	13.38	96.7
			0.6	14.60	13.40	96.9
Chlorella protein* 100 mg.	10.76	9.86	0.4	9.67	9.07	92.0
			0.6	10.05	9.14	92.7
Scenedesmus protein** 100 mg.	10.78	10.00	0.4	10.00	9.14	91.4
			0.6	10.28	9.31	93.1
Dried chlorella 100 mg.	9.74	9.08	0.4	3.17	2.51	27.6
			0.6	3.21	2.49	27.4
Decolored chlorella 100 mg.	7.70	7.09	0.4	5.92	5.32	75.1
			0.6	6.97	5.24	74.0
Lyophilized chlorella 100 mg.	10.15	9.50	0.4	6.13	5.22	57.1
			0.6	6.45	5.67	59.7

* Isolated by the autolysis-butanol treatment. (Table 1, No. 17).

** Isolated by the altered autolysis-butanol treatment. (Scheme 2, Table 3).

TCA : Trichloroacetic acid.

temperature of algae was a little higher, the algae seemed to be autolyzed, and the protein might be digested.

The digestibility of the cell free protein by trypsin were summarized in Table 5.

All of the cell free protein indicated good digestibility, that is, the value was match for that obtained with purified casein. Dried chlorella indicated very low digestibility, while the hot methanol treatment chlorella had considerable availability.

The digestibilties and biological values with rats are summarized in Table 6. The digestibility of cell free protein was a little lower than that with trypsin, although it was much higher than that of dried chlorella with rats.

The biological value of cell free algal protein was lower than that of

Table 6. The digestibility and Biological Value of Isolated Cell Free Scenedesmus Protein with Rat *in vivo*.

Group	Rat No	Original weight of rat	Daily weight gaine of rat	Daily N intake	Daily fecal N	Daily urinary N	Metabolic N in feces	Endogenous N	Food N in feces	Food N in urine	Absorbed N	Retained N	Digestibility	Biological value	Net protein value
		(g.)	(g.)	(mg.)	(mg.)	(mg.)	(mg.)	(mg.)	(mg.)	(mg.)	(mg.)	(mg.)	(%)		
Casein diet	11	169	1.8	169.7	17.5	47.9	13.2	30.5	4.3	17.4	165.4	148.0	97.5	89.5	7.18
	12	201	0.9	185.6	21.6	59.5	14.6	33.4	7.0	26.5	178.6	152.1	96.3	85.3	2.28
	13	227	1.5	183.0	19.5	41.1	10.0	29.1	9.5	12.0	173.0	161.0	94.8	93.0	8.05
Isolated cell free scenedesmus protein diet	21	168	2.5	236.0	44.1	105.1	14.4	37.9	29.7	67.2	206.3	139.1	87.4	67.5	59.0
	22	179	3.3	200.0	40.1	113.7	12.4	42.2	27.7	71.5	172.3	100.8	86.2	58.5	50.4
	23	223	1.2	202.0	47.2	123.8	18.1	48.9	29.1	74.9	172.9	98.0	85.6	56.8	48.7
Dried chlorella diet	31	193	1.6	184.0	29.7	89.9	16.0	32.9	81.2	57.0	102.8	45.8	55.2	44.6	24.6
	32	206	0.6	227.0	129.5	100.2	20.8	33.8	108.7	66.4	108.3	51.9	47.8	47.9	22.9
	33	209	0.1	214.0	119.7	100.8	30.0	46.0	73.7	54.8	140.3	85.5	65.5	60.5	39.6

dried chlorella. However, the net protein values (*i. e.* Biological value × Digestibility) of the cell free protein was much higher than that of dried chlorella.

Discussion

The results obtained by the digestion test with trypsin and rats indicated that the isolated cell free algal proteins had much higher digestibilities which were match for that of casein.

The comparison of these results with those reported by other workers[15) 16)] are presented in Table 7.

Although the digestibility of algal protein is increased a little by the hot methanol treatment, it is still not a satisfactory human food. Moreover, the

Table 7. The comparison of digestibilities of chlorella protein reported several workers.

	Digestibility with trypsin *in vitro*		
Casein	96.9	99.5	91.8***
Cell free chlorella protein*	92.7		
Cell free scenedesmus protein**	93.1		
Dried chlorella	27.4	62.5~65.5	43.9***
Decolored chlorella	75.1	26.3	
Lyophilized chlorella	59.7	27.6	
Fresh chlorella		46.2	
	Digestibility with rat *in vivo*		
Casein	94.8~94.7	95.9~98.0	
Cell free scenedesmus protein**	85.6~87.4		
Dried chlorella	47.8~65.5	49.1~58.4	67.0~72.0
Decolored chlorella		77.1~84.4	
Decolored scenedesmus		66.6~73.0	
Reference	present authors	(15)	(16)

* Isolated by the autolysis-butanol treatment. (Table 1, No. 17).
** Isolated by the altered autolysis-butonol treatment. (Scheme, 2, Table 73)
*** The value with pepsin digestion.

indigestable materials such as cell wall and so on were not removed. Therefore, the hot methanol treated algae can not be used for human food.

The biological value of isolated cell free protein is lower than that of casein. It seems that the amino acid composition of algal protein is inferior to that of casein. The amino acid composition of the isolated cell free protein is under investigation. Generally, the algal proteins contained poorly sulfur containing amino acids[17)].

As the sulfur containing amino acids are essential for animal nutrition, these amino acids are a limiting amino acid in algal proteins, and thus the biological values of them are restricted.

It was of interest that the biological value of isolated cell free protein

was lower than that of casein, but the protein was superior to casein in the restoration of body weights of rats. The increase in the body weight depends not only upon the increase in tissue protein but also upon many other factors. Therefore, a protein which is superior in biological value is not always effective in increasing the body weight. On the other hand, H. Fink[10)11)] reported that the algal protein was much effective to the growth of young rats, that is, its nutritive value was match for that of casein. Therefore, the presence of unknown active substance which might be contained in the isolated cell free protein preparation can be expected. In regard to this problem, further investigations are necessary.

The biological value is not influenced by digestibility of the protein. The value depends upon the protein score, that is, essential amino acid composition. But, this principle is due to a premise that the protein is digestable enough. The algal protein is not single, but consists of several proteins which are different to each other in amino acid compositions and in chemical and physical natures. Moreover, their states and sites in the cell were different. Hence, the biological value might be affected by the digestibility as in the cases of the dried chlorella and the hot methanol treated chlorella.

III. THE AVAILABILITY OF ALGAL PROTEIN FOR FOOD PROTEIN

The nutritive value of algal protein itself is not very high ; this is suggested by its amino acid composition and the biological value obtained with rat. However, the fact that the algal protein is rich in lysine, threonine and tryptophan, the content of which in cereal proteins are relatively low, suggests that it can be used to cover the weak point of the cereal protein. An attempt to clarify this problem by using the circular explanatogram of limiting amino acid, which is devised by the authors[18)], are presented.

The nutritive value of protein depends upon its amino acid composition,

Table 8. The minimum requirements of essential amino acids.

	Minimum requirments (g./day) Man[27)]	Woman[23),29)]	Safe intakes of man[27)] (g./day)	Maintenance pattern for man	Minimum requirement of infant[30)] (mg./kg.)	Growth pattern	Maintenance pattern of FAO[33)]
Tryptophan	0.25	0.16	0.50	1.0	30	1.0	1.0
Phenylalanine	1.10*	1.12	2.20	4.4	90**	3.0	2.8
Lysine	0.80	0.40	1.60	3.2	90	3.0	3.2
Threonine	0.50	0.31	1.00	2.0	60	2.0	2.0
Valine	0.80	0.65	1.60	3.2	85	2.8	4.0
Methionine	1.10***	0.49	2.20	4.4	85****	2.8	2.8
Leucine	1.10	0.62	2.20	4.4			4.0
Isoleucine	0.70	0.45	1.40	2.8	90	3.0	3.0

* 70% of the requirement can be replaced by tyrosine[31)].

** In presence of tyrosine.

*** 80% of the requirement can be replaced by cystine[32)].

**** 65 mg./kg. in presence of cystine.

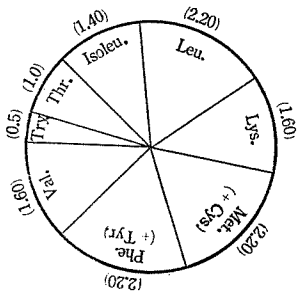
and particularly upon that of essential amino acids. Furthermore it has been recognized by many investigators[19-23] that the maximum manifestation of the nutritive efficiency of each essential amino acid should be present at a certain definite proportion (amino acid pattern) as well as in large quantity. It has been agreed that the ratio of the minimum requirements of the essential amino acids can be adopted as the optimal proportion[24)25)].

The essential amino acids for human being were decided by Rose[26] ; the minimum requirements are shown in Table 8. Although it is not too surprising that the minimum requirements depend on age and sex, it is interesting to note that the ratios of the minimum requirements are essentially constant. The standard proportion has been proposed by FAO[33] as indicated in Table 8. The minimum requirements are literally the minimum amounts necessary to maintain the nitrogen balance, and it is obvious that the more amount is necessary for the maintenance of healthy life. As to this amount we have not sufficient experimental knowledge at present, and most of the discussion is based merely on practical experience. W. C. Rose[27] has proposed the double amount of the minimal requirment of each essential amino acid as the safe intake based on a number of his nitrogen balance tests with adult men. On the other hand, M. Bricker *et. al*[34]. reported that 74.4 g. per day of wheat protein or 43 g. per day of milk protein were required for the satisfactory maintenance of body nitrogen of an adult female weighing 70 kg. The quantities of the essential amino acids contained in the proposed amounts of these proteins can be calculated and turn out that the amount of the limiting amino acid is close to the value of Rose's safe intake. Lysine is limited in the case of wheat protein and its amount corresponds to 106 per cent of Rose's safe intake, while the contents of sulfur containing amino acids, which are limited in the case of milk protein, amount to 68 per cent of the safe intake. Generally the minimum requirements of essential amino acids for female are lower than those for male. Therefore, Rose's safe intakes can be considered as reasonable values for adult with rather wide allowance.

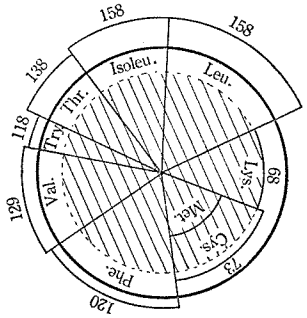
From the above discussion it is clear that, for the tabulation of the nutritive value of protein by considering the amino acid contents, one must take into consideration the two factors, namely the contents of individual essential amino acids and their proportion, and then, combine these two elements in a single and clear-cut indication. For this purpose the present authors have devised a method to indicate the limiting amino acid of a protein in a very simple fashion as will be explained in the following, and has named it circular explanatogram of limiting amino acid.

The center angle of a circular diagram with a certain radius is divided in proportion to the amounts of the minimum requirements of the essential amino acids. Thus the areas of the resulting eight semicircles are in proportion to the ratios of the amounts of the essential amino acids, which allow the manifestation of the maximum nutritive efficiency. When the eight semicircles with areas corresponding to the standard intake of each essential amino acids are put together, they form a complete circle with a certain

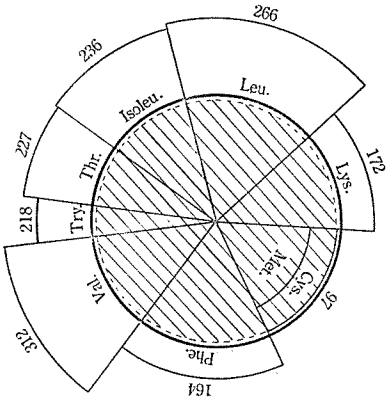
definite radius which represents the standard intakes of the essential amino acids. In Fig. 3-A is shown such a circle by using Rose's safe intakes as the standard.



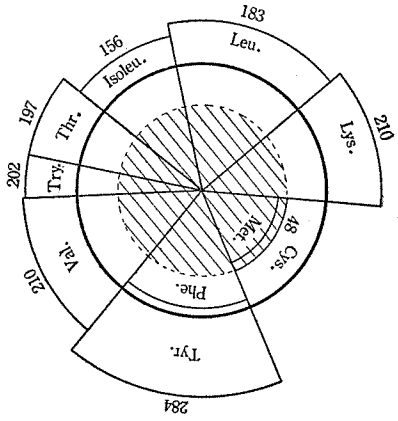
(A) Standard. (Rose's safe intakes)



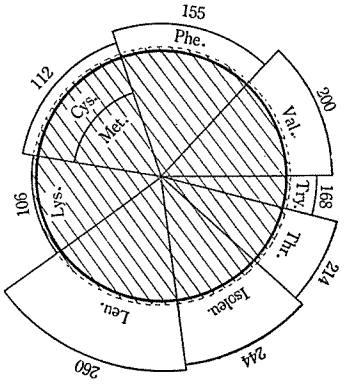
(B) 600 g. of bread. (protein 48.0 g.)*



(C) Chlorella protein. (48.0 g.)



(D) 600 g. of bread supplemented by 4% of chlorella protein. (protein 72.0 g.)



(E) Wheat protein. (74.4 g.)*

Figure 3. The circular explanatograms of limiting amino acids of several proteins.

* Amino acid composition was calculated from Orr's data (35).
Numbers in parentheses in (A) show the safe intakes of each amino acids (g./day).
Numbers in (B) to (E) show the propotions of amounts of each amino acids to the safe intakes (%).

In order to represent the nutritive value of a certain amount of a given protein, a semicircle for each essential amino acid with the same central angle as that in Fig. 3-A and with area corresponding to its content in the given protein is drawn. Consequently the radii of these eight semicircles are proportional to the contents of these amino acids in the protein and hence are not uniform in general cases. Thus the resulting circular diagram consists of indented and projectile parts. An example is shown in Fig. 3-B for 600 g. of bread prepared from 480 g. of flour. The most efficient amino acid composition should give the diagram with a smooth periphery or a complete circle.

In the Present example the amounts of amino acids contained in the largest possible complete circle, that is the one with radius of the semicircle for lysine (covered oblique lines), are the part which is utilized most efficiently. The amount of this part contained in 600 g. of bread corresponds to approximately 68 per cent of the safe intakes. Thus the content of lysine in wheat protein is the lowest and limits the nutritive value of the protein. Therefore the utilization of other amino acids is extremely inefficient, for example, the efficiencies of phenylalanine, threonine, valine, and isoleucine are as low as 50 per cent or less. For this example, it can eassily be seen that the smoother the diagram the better is the quality of a protein. In wheat protein, lysine and sulfur containing amino acids, *i.e.* methionine and cystine, are scarce. On the other hand, the amino acid composition of bulk protein of chlorella[12] is well balanced except for the sulfur containing amino acid, as is represented in Fig. 3-C. Comparing the nutritive value of protein in the same quantity, the protein contained in bread is superior to chlorella protein as clearly shown by the area of the complete circle in Figs. 3-B and 3-C. If one supplement the 600 g. of bread with chlorella protein at the proportion of 5 g. for each 100 g. of wheat flour, the diagram represented in Fig. 3-D is obtained. It clearly shows a marked improvement in the amino acid composition to make the combined ration is sufficient to cover the safe intakes. Although 74.4 g. of wheat protein (Fig. 3-E) or approximately 930 g. of bread are required to cover the safe intakes, an addition of only 4 per cent of chlorella protein can reduce the required amount of bread to 600 g.

From the above discussion it can be concluded that algal protein, like that of chlorella, can be utilized with remarkable results as a supplement to wheat flour replacing defatted milk powder, which is now being in use, or as an additive to soybean meal to be prepared to many kinds of food. Consequently in utilizing the algal protein, it should be so prepared as to be suitable for versatile use in food technology. At present only decolored chlorella from hot methanol treatment or simply heat treated chlorella is used as food. The authors believe that this quite unsatisfactory state should be improved so that the algal protein, as well as other precious ingredient like chlorophyll, carotine, folic acid, vitamin B_{12} *etc.* can be utilized with more profit by isolating these component before use, and then applying separately in the most efficient way.

SUMMARY

1) The destraction of algal cell wall by the mechanical treatment such as grinding, freezing and so on, was effective to extract the nitrogenous cell materials.

2) Alkakine extraction was very effective in extraction of algal nitrogenous component. But since the protein obtained had thickly dark greenish color, it was unsuitable for food. However, the addition of some detergents into alkaline solution prevented the coloring of the extracted algal protein. A further investigation on this subject is now being conducted.

3) A considerable amount of algal nitrogenous material was extracted by means of autolysis, but the yield of protein was not always high.

4) Butanol treatment was effective in fractional isolation of protein, non-protein-nitrogenous materials and pigments simultaneously.

5) The combination of autolysis and butanol treatment is one of the most effective ways for extraction of algal nitrogenous materials. That is, 60 per cent of algal total nitrogen was extracted, and the protein obtained corresponded to 30 per cent of algal true protein.

6) The weak point of the butanol treatment, that is, the procedure with ethanol-ether mixture, was excluded by the use of grinding procedure instead of that procedure.

7) By the altered autolysis-butanol treatment, 120 g. of cell free protein were prepared from 4.7 kg. of fresh scenedesmus.

8) The digestibility of isolated cell free algal protein was 91-93 per cent *in vitro* with trypsine, and 76-81 per cent *in vivo* with rat. These values are match for that of casein, and are excellent for that of dried chlorella or decolored chlorella from hot methanol treatment.

9) The biological value of isolated cell free protein for rat was 57-68. Although it was considerably inferior to that of casein, it was a little superior to that of dried chlorella. But the net protein value of the isolated protein is twice of that of dried chlorella.

10) The nutritive value of chlorella protein is low when it is used independently. But the protein is available to use for the improvement of the nutritive value of cereal proteins. This was explained by the use of the circular explanatogram of limiting amino acid which was devised by the authors.

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REFERENCES

(1) J. S. Burlew, "Algal calture from laboratory to pilot plant," p.iii. Carnegie Institution of Washington Publication 600, Washington D. C., (1953).
(2) J. S. Burlew, *ibid.* p. iii ; R. Emerson, J. F. Stauffer, and W. W. Umbreit, *Amer. Jour. Bot.*, **31**, 107 (1944).
(3) H. A. Spoehr *et al.*, "Fatty acid antibacterials from plants," Carnegie Inst. of Washington Pub. 586 (1949).
(4) H. A. Spoehr, and H. W. Millner, *Plant. Physiol.*, **24**, 120 (1949).
(5) H. W. Millner, *J. Biol. Chem.*, **176**, 813 (1948).
(6) H. A. Spoehr, and H. W. Millner, Carnegie Inst. of Washington Year Book No .47, 100 (1948).
(7) H. Tamiya, *et al.*, "Algal culture from laboratory to pilot plant," p. 204. Carnegie Inst. of Washington Pub. 600, Washington, D. C., (1953).
(8) A. Mituya, T. Nyunoya, and H. Tamiya, *ibid*, p. 273.
(9) H. Tamiya, *Ann. Rev. Plant Physiol.*, 8, 309 (1957).
(10) H. Fink, *Biochem. Z.*, **323**, 389 (1953).
(11) H. Fink, *Naturwissench.*, **41**, 169 (194).
(12) L. Fowden, *Biochem.J.*, **50**, 355 (1951).
(13) D. H. Northcote, *et al.*, *Biochem. J.*, **70** 391 (1958).
(14) H. H. Mitchel., *J. Biol. Chem.*, **58**, 873 (1924).
(15) E. Tamura, *et al.*, "Report of the Research Council for the Studies on Essential Amino Acid," March, 1958 ; *ibid.* May, 1958.
(16) M. Kandachi, *et al.*, *ibid.* March, 1957; *ibid.* March, 1958.
(17) R. J. Block, and K. W. Weiss, "Amino Acid Hand Book, Methods and results of protein analysis," p. 323. Charles C. Thomas Publisher, Springfield, Illinois (1956).
(18) H. Mitsuda, Reported on 60th Semi-Annual Meeting of the Inst. for Chem. Res. of Kyoto Univ. on 6th, Dec., 1958. ; H. Mitsuda, "The Basics of Nutritional Chemistry" (Japanese), p. 168, Sangyo Toshyo Co., Kanda. Tokyo (1959).
(19) H. J. Almquist, *Proc. Soc. Exp. Biol. Med.*, **72**, 179 (1949).
(20) H. J. Almquist, *Arch. Biochem. Biophys.*, **46**, 250 (1953).
(21) R. Bressani, and E. T. Mertz, *Fed. Proc.*, 15, 544 (1956).
(22) P. Griminger, H. M. Scott and R. M. Forbes, *J. Nutrition*, **59**, 67 (1956).
(23) M. Recheigl, Jr. *et al.*, *J. Nutrition*, **60**, 619 (1956).
(24) J. K. Loosli, "Proc. 1950, Cornell Nutrition Conf. for Food Mfrs.," p. 30.
(25) W. W. Beeson, *J. Lab. Clin. Med.*, **33**, 47 (1948).
(26) W. C. Rose, *et al.*, *J. Biol. Chem.*, **182**, 541 (1950) ; **188**, 49 (1951) ; **193**, 605, 613 (1951) ; **206**, 421 (1954).
(27) W. C. Rose, *et al.*, *J. Biol. Chem.*, **211**, 815 (1954) ; **213**, 913 (1955) ; **214**, 579 (1955) ; **215**, 101 (1955) ; **216**, 225 (1955).
(28) W. H. Grifith, and M. E. Swendseid, *Ann. Rev. of Biochem.*, **25**, 537 (1956).
(29) R. M. Leverton, *et al.*, *J. Nutrition*, **85**, 59, 83, 219, 341, 355 (1956).
(30) E. L. Pratt, *J. Amer. Med. Assoc.*, **164**, 408 (1957).
(31) W. C. Rose, *et al.*, *J. Biol. Chem.*, **217**, 95 (1955).
(32) W. C. Rose, *et al.*, *S. Biol. Chem.*, **216** ; 763 (1955).
(33) FAO Nutrition Studies, No. 16. "Protein Requirements" Report of the FAO Committee, Rome, Italy, 24-31 October 1955. FAO Rome. 1957.
(34) M. Bricker, H. H. Mitchel, and G. M. Kinsman, *J. Nutrition*, **30**, 269 (1945).
(35) M. L. Orr and B. K. Watt, "Amino acid content of foods," (1957).